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Invention: USE OF FACTOR X POLYMORPHISM IN THE DIAGNOSIS AND TREATMENT OF FACTOR X AND/OR FACTOR XA MEDIATED DISEASES

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SPECIFICATION

USE OF FACTOR X POLYMORPHISM IN THE DIAGNOSIS AND TREATMENT OF FACTOR X AND/OR FACTOR XA MEDIATED DISEASES

This invention relates to polymorphisms in the Factor X gene. The invention also relates to methods and materials for analysing allelic variation in the Factor X gene, and to the use of Factor X polymorphism in the diagnosis and treatment of Factor X and/or Factor Xa-mediated diseases, such as thrombotic diseases.

Factor Xa is one of a cascade of proteases involved in the complex process of blood coagulation. The protease known as thrombin is the final protease in the cascade and Factor Xa is the preceding protease which cleaves prothrombin to generate thrombin. Factor Xa is produced by cleavage of the zymogen precursor Factor X, by activated factor VII. For a review of the process of blood coagulation see Rock and Wells (1997) Crit Rev Clin Lab Sci **34**, 475-501 and for a review of the Biochemistry of Factor X see Hertzberg (1994) Blood Reviews 8, 56-62.

Certain compounds are known to possess Factor Xa inhibitory properties and the field has been reviewed by R.B. Wallis, Current Opinion in Therapeutic Patents, 1993, 1173-1179 and Yamazaki (1995) Drugs of the Future 20, 911-918. Thus it is known that two proteins, one known as antistatin and the other known as tick anticoagulant protein (TAP), are specific Factor Xa inhibitors which possess antithrombotic properties in various animal models of thrombotic disease.

It is also known that certain non-peptidic compounds possess Factor Xa inhibitory properties. Of the low molecular weight inhibitors mentioned in the review by R.B. Wallis, all possessed a strongly basic group such as an amidinophenyl or amidinonaphthyl group.

The sequence of Factor X was published by Leytus et al (1986) Biochemistry **25**, 5098-5102. The sequence was submitted to the EMBL database as separate exons: Exon 1 (EMBL Accession Number -L00390), Exon 2 (EMBL Accession Number - L00391), Exon 3 (EMBL Accession Number - L00392), Exon 4 (EMBL Accession Number - L00393), Exon 5 (EMBL Accession Number - L00394), Exon 6 ((EMBL Accession Number - L00395), Exon 7 (EMBL Accession Number - L00396), and Exon 8 (EMBL Accession Number - L29433). All positions herein relate to the position in the appropriate EMBL Accession number unless stated otherwise or apparent from the context.

Mutations in the Factor X gene which lead to Factor X deficiency and a clinical phenotype are well documented (For a review of Factor X mutations and Factor X deficiency see Cooper et al (1997) Thrombosis and Haemostasis 78, 161-172).

Other variation in DNA sequence (polymorphisms) may not lead to Factor X deficiency but may increase the probability of pathological conditions or affect drug response or may be genetically linked to other polymorphisms which do so.

One approach is to use knowledge of polymorphisms to help identify patients most suited to therapy with particular pharmaceutical agents (this is often termed "pharmacogenetics") . Pharmacogenetics can also be used in pharmaceutical research to assist the drug selection process. Polymorphisms are used in mapping the human genome and to elucidate the genetic component of diseases. The reader is directed to the following references for background details on pharmacogenetics and other uses of polymorphism detection: Linder *et al.* (1997), Clinical Chemistry, **43**, 254; Marshall (1997), Nature Biotechnology, **15**, 1249; International Patent Application WO 97/40462, Spectra Biomedical; and Schafer *et al.* (1998), Nature Biotechnology, **16**, 33.

Clinical trials have shown that patient response to treatment with pharmaceuticals is often heterogeneous. Thus there is a need for improved approaches to pharmaceutical agent design and therapy.

The present invention is based on the discovery of two single nucleotide polymorphisms (SNPs) in the coding sequence of the human Factor X gene.

According to one aspect of the present invention there is provided a method for the diagnosis of a single nucleotide polymorphism in a Factor X gene in a human, which method comprises determining the sequence of the nucleic acid of the human

at position 41 in exon 5 of the Factor X gene as defined by the position in EMBL ACCESSION NO. L00394, and/or

at position 57 in exon 7 of the Factor X gene as defined by the position in EMBL ACCESSION NO. L00396 and determining the status of the human by reference to polymorphism in the Factor X gene.

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at position 57 in exon 7 of the Factor X gene as defined by the position in EMBL ACCESSION NO. L00396 and determining the status of the human by reference to polymorphism in the Factor X gene.

The term human includes both a human having or suspected of having a Factor X-mediated disease and an asymptomatic human who may be tested for predisposition or susceptibility to such disease. At each position the human may be homozygous for an allele or the human may be a heterozygote.

In one embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at exon 5 position 41 is presence of C and/or T.

In another embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at exon 7 position 57 is presence of C and/or T.

Subsequently to the present invention, Cargill et al have confirmed the presence of a single nucleotide polymorphism in human Factor X at exon 5 position 41 and/or at exon 7 position 57 (Cargill et al., Nature Genetics, 22, 231-239, 1999).

The method for diagnosis is preferably one in which the sequence is determined by a method selected from amplification refractory mutation system and restriction fragment length polymorphism.

In another aspect of the invention we provide a method for the diagnosis of Factor X- and/or Factor Xa-mediated disease, which method comprises:

i) obtaining sample nucleic acid from an individual,

ii) detecting the presence or absence of a variant nucleotide at position 41 in exon 5 of the Factor X gene as defined by the position in EMBL ACCESSION NO. L00394, and/or at position 57 in exon 7 of the Factor X gene as defined by the position in EMBL ACCESSION NO. L00396,

iii) determining the status of the individual by reference to polymorphism in the Factor X gene.

Allelic variation at exon 5 position 41 consists of a single base substitution from C (the published base), preferably to T. Allelic variation at exon 7 position 57 consists of a single base substitution from C (the published base), preferably to T.

The status of the individual may be determined by reference to allelic variation at any one or both positions optionally in combination with any other polymorphism that is or becomes known.

The test sample of nucleic acid is conveniently a sample of blood, bronchoalveolar lavage fluid, sputum, or other body fluid or tissue obtained from an individual. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample, that is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique e.g. PCR, before analysis of allelic variation.

It will be apparent to the person skilled in the art that there are a large number of analytical procedures which may be used to detect the presence or absence of variant nucleotides at one or more polymorphic positions of the invention. In general, the detection of allelic variation requires a mutation discrimination technique, optionally an amplification reaction and optionally a signal generation system. Table 1 lists a number of mutation detection techniques, some based on the PCR. These may be used in combination with a number of signal generation systems, a selection of which is listed in Table 2. Further amplification techniques are listed in Table 3. Many current methods for the detection of allelic variation are reviewed by Nollau *et al.*, Clin. Chem. **43**, 1114-1120, 1997; and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

Abbreviations:

ALEX™	Amplification refractory mutation system linear extension
APEX	Arrayed primer extension
ARMS™	Amplification refractory mutation system
b-DNA	Branched DNA
CMC	Chemical mismatch cleavage
bp	base pair

COPS	Competitive oligonucleotide priming system
DGGE	Denaturing gradient gel electrophoresis
FRET	Fluorescence resonance energy transfer
LCR	Ligase chain reaction
MASDA	Multiple allele specific diagnostic assay
NASBA	Nucleic acid sequence based amplification
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PTT	Protein truncation test
RFLP	Restriction fragment length polymorphism
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
SSR	Self sustained replication
TGGE	Temperature gradient gel electrophoresis

Table 1 - Mutation Detection Techniques

General: DNA sequencing, Sequencing by hybridisation

Scanning: PTT*, SSCP, DGGE, TGGE, Cleavase, Heteroduplex analysis, CMC, Enzymatic mismatch cleavage

* Note: not useful for detection of promoter polymorphisms.

Hybridisation Based

Solid phase hybridisation: Dot blots, MASDA, Reverse dot blots, Oligonucleotide arrays (DNA Chips)

Solution phase hybridisation: Taqman™ - US-5210015 & US-5487972 (Hoffmann-La Roche), Molecular Beacons - Tyagi *et al* (1996), Nature Biotechnology, **14**, 303; WO 95/13399 (Public Health Inst., New York)

Extension Based: ARMS™, ALEX™ - European Patent No. EP 332435 B1 (Zeneca Limited), COPS - Gibbs *et al* (1989), Nucleic Acids Research, **17**, 2347.

Incorporation Based: Mini-sequencing, APEX

Restriction Enzyme Based: RFLP, Restriction site generating PCR

Ligation Based: OLA

Other: Invader assay

Table 2 - Signal Generation or Detection Systems

Fluorescence: FRET, Fluorescence quenching, Fluorescence polarisation - United Kingdom Patent No. 2228998 (Zeneca Limited)

Other: Chemiluminescence, Electrochemiluminescence, Raman, Radioactivity, Colorimetric, Hybridisation protection assay, Mass spectrometry

Table 3 - Further Amplification Methods

SSR, NASBA, LCR, SDA, b-DNA

Preferred mutation detection techniques include ARMS™, ALEX™, COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

Particularly preferred methods include ARMS™ and RFLP based methods. ARMS™ is an especially preferred method.

In a further aspect, the diagnostic methods of the invention are used to assess the efficacy of therapeutic compounds in the treatment of Factor X and/or Factor Xa-mediated diseases, such as thrombotic diseases.

Assays, for example reporter-based assays, may be devised to detect whether one or more of the above polymorphisms affect transcription levels and/or message stability.

Individuals who carry particular allelic variants of the Factor X gene may therefore exhibit differences in their ability to regulate protein biosynthesis under different physiological conditions and will display altered abilities to react to different diseases. In addition, differences in protein regulation arising as a result of allelic variation may have a direct effect on the response of an individual to drug therapy. The diagnostic methods of the invention may be useful both to predict the clinical response to such agents and to determine therapeutic dose.

In a further aspect, the diagnostic methods of the invention, are used to assess the predisposition of an individual to diseases mediated by Factor X and/or Factor Xa. This may be particularly relevant in the development of thrombotic disease and other diseases which are

modulated by Factor X and/or Factor Xa. The present invention may be used to recognise individuals who are particularly at risk from developing these conditions.

Low frequency polymorphisms may be particularly useful for haplotyping as described below. A haplotype is a set of alleles found at linked polymorphic sites (such as within a gene) on a single (paternal or maternal) chromosome. If recombination within the gene is random, there may be as many as 2^n haplotypes, where 2 is the number of alleles at each SNP and n is the number of SNPs. One approach to identifying mutations or polymorphisms which are correlated with clinical response is to carry out an association study using all the haplotypes that can be identified in the population of interest. The frequency of each haplotype is limited by the frequency of its rarest allele, so that SNPs with low frequency alleles are particularly useful as markers of low frequency haplotypes. As particular mutations or polymorphisms associated with certain clinical features, such as adverse or abnormal events, are likely to be of low frequency within the population, low frequency SNPs may be particularly useful in identifying these mutations (for examples see: Linkage disequilibrium at the cystathionine beta synthase (CBS) locus and the association between genetic variation at the CBS locus and plasma levels of homocysteine. *Ann Hum Genet* (1998) **62**:481-90, De Stefano V, Dekou V, Nicaud V, Chasse JF, London J, Stansbie D, Humphries SE, and Gudnason V; and Variation at the von willebrand factor (vWF) gene locus is associated with plasma vWF:Ag levels: identification of three novel single nucleotide polymorphisms in the vWF gene promoter. *Blood* (1999) 93:4277-83, Keightley AM, Lam YM, Brady JN, Cameron CL, Lillicrap D).

In a further aspect, the diagnostic methods of the invention are used in the development of new drug therapies which selectively target one or more allelic variants of the Factor X gene. Identification of a link between a particular allelic variant and predisposition to disease development or response to drug therapy may have a significant impact on the design of new drugs. Drugs may be designed to regulate the biological activity of variants implicated in the disease process whilst minimising effects on other variants.

In a further diagnostic aspect of the invention the presence or absence of variant nucleotides is detected by reference to the loss or gain of, optionally engineered, sites recognised by restriction enzymes. In the accompanying Example 2 we provide details of convenient engineered restriction enzyme sites that are lost or gained as a result of a polymorphism of the invention.

According to another aspect of the present invention there is provided a nucleic acid comprising any one of the following polymorphisms:

the nucleic acid of EMBL ACCESSION No. L00394 with T at position 41 as defined by the position in EMBL ACCESSION No. L00394;

the nucleic acid of EMBL ACCESSION No. L00396 with T at position 57 as defined by the position in EMBL ACCESSION No. L00396;

or a complementary strand thereof or an antisense sequence thereto or a fragment thereof of at least 20 bases comprising at least one polymorphism.

Fragments are at least 17 bases, more preferably at least 20 bases, more preferably at least 30 bases.

Novel sequence disclosed herein, may be used in another embodiment of the invention to regulate expression of the gene in cells by the use of antisense constructs. To enable methods of down-regulating expression of the gene of the present invention in mammalian cells, an example antisense expression construct can be readily constructed for instance using the pREP10 vector (Invitrogen Corporation). Transcripts are expected to inhibit translation of the gene in cells transfected with this type construct. Antisense transcripts are effective for inhibiting translation of the native gene transcript, and capable of inducing the effects (e.g., regulation of tissue physiology) herein described. Oligonucleotides which are complementary to and hybridizable with any portion of novel gene mRNA disclosed herein are contemplated for therapeutic use. U.S. Patent No. 5,639,595, Identification of Novel Drugs and Reagents, issued Jun. 17, 1997, wherein methods of identifying oligonucleotide sequences that display in vivo activity are thoroughly described, is herein incorporated by reference. Expression vectors containing random oligonucleotide sequences derived from previously known polynucleotides are transformed into cells. The cells are then assayed for a phenotype resulting from the desired activity of the oligonucleotide. Once cells with the desired phenotype have been identified, the sequence of the oligonucleotide having the desired activity can be identified. Identification may be accomplished by recovering the vector or by polymerase chain reaction (PCR) amplification and sequencing the region containing the inserted nucleic acid material.nucleotide molecules can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-O-alkylRNA, or other oligonucleotide mimetics. U.S. Patent No. 5,652,355, Hybrid

Oligonucleotide Phosphorothioates, issued July 29, 1997, and U.S. Patent No. 5,652,356, Inverted Chimeric and Hybrid Oligonucleotides, issued July 29, 1997, which describe the synthesis and effect of physiologically-stable antisense molecules, are incorporated by reference. Antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence.

The invention further provides nucleotide primers which can detect the polymorphisms of the invention.

According to another aspect of the present invention there is provided an allele specific primer capable of detecting a Factor X gene polymorphism

at position 41 in exon 5 of the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00394, and/or

at position 57 in exon 7 in the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00396.

An allele specific primer is used, generally together with a constant primer, in an amplification reaction such as a PCR reaction, which provides the discrimination between alleles through selective amplification of one allele at a particular sequence position e.g. as used for ARMS™ assays. The allele specific primer is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.

An allele specific primer preferably corresponds exactly with the allele to be detected but derivatives thereof are also contemplated wherein about 6-8 of the nucleotides at the 3' terminus correspond with the allele to be detected and wherein up to 10, such as up to 8, 6, 4, 2, or 1 of the remaining nucleotides may be varied without significantly affecting the properties of the primer.

Primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition. If required the primer(s) may be labelled to facilitate detection.

According to another aspect of the present invention there is provided an allele-specific oligonucleotide probe capable of detecting a Factor X gene polymorphism

at position 41 in exon 5 of the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00394, and/or

at position 57 in exon 7 in the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00396.

The allele-specific oligonucleotide probe is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.

According to another aspect of the present invention there is provided a diagnostic kit comprising an allele specific oligonucleotide probe of the invention and/or an allele-specific primer of the invention.

The diagnostic kits may comprise appropriate packaging and instructions for use in the methods of the invention. Such kits may further comprise appropriate buffer(s) and polymerase(s) such as thermostable polymerases, for example taq polymerase.

In another aspect of the invention, the single nucleotide polymorphisms of this invention may be used as genetic markers in linkage studies. This particularly applies to the polymorphism at exon 7 position 57 because of its informative frequency (see below). The Factor X gene has been mapped to chromosome 13q34 (Bowcock et al, Genomics **16**, 486-496, 1993).

According to another aspect of the present invention there is provided a method of treating a human in need of treatment with a Factor Xa ligand antagonist drug in which the method comprises:

i) diagnosis of a single nucleotide polymorphism in Factor X gene in the human, which diagnosis comprises determining the sequence of the nucleic acid

at position 41 in exon 5 of the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00394, and/or

at position 57 in exon 7 in the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00396.

and determining the status of the human by reference to polymorphism in the Factor X gene; and

ii) administering an effective amount of a Factor Xa ligand antagonist drug.

The term " Factor Xa ligand antagonist drug" includes drugs acting at Factor Xa and/or Factor X but the former is preferred.

Factor Xa ligand antagonist drugs possess activity in the treatment or prevention of a variety of medical disorders where anticoagulant therapy is indicated, for example in the treatment or prevention of thrombotic conditions such as coronary artery and cerebro-vascular disease. Further examples of such medical disorders include various cardiovascular and cerebrovascular conditions such as myocardial infarction, the formation of atherosclerotic plaques, venous or arterial thrombosis, coagulation syndromes, vascular injury including reocclusion and restenosis following angioplasty and coronary artery bypass surgery, thrombus formation after the application of blood vessel operative techniques or after general surgery such as hip replacement surgery, the introduction of artificial heart valves or on the recirculation of blood, cerebral infarction, cerebral thrombosis, stroke, cerebral embolism, pulmonary embolism, ischaemia and angina (including unstable angina).

Preferably determination of the status of the human is clinically useful. Examples of clinical usefulness include deciding which antagonist drug or drugs to administer and/or in deciding on the effective amount of the drug or drugs.

Inhibitors of Factor Xa have been disclosed in the following publications: European patent application EP 540051 A, Daiichi; WO98/21188, Zeneca Ltd and WO96/10022, Zeneca Ltd.

According to another aspect of the present invention there is provided use of a Factor Xa ligand antagonist drug in preparation of a medicament for treating a Factor Xa and/or Factor X-mediated disease in a human diagnosed as having a single nucleotide polymorphism at position 41 in exon 5 of the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00394, and/or
at position 57 in exon 7 in the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00396.

According to another aspect of the present invention there is provided a pharmaceutical pack comprising a Factor Xa-ligand antagonist drug and instructions for administration of the drug to humans diagnostically tested for a single nucleotide polymorphism

at position 41 in exon 5 of the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00394, and/or

at position 57 in exon 7 in the Factor X gene as defined by the positions in EMBL ACCESSION NO. L00396.

According to another aspect of the present invention there is provided a computer readable medium comprising at least one novel polynucleotide sequence of the invention stored on the medium. The computer readable medium may be used, for example, in homology searching, mapping, haplotyping, genotyping or pharmacogenetic analysis or any other bioinformatic analysis. The reader is referred to Bioinformatics, A practical guide to the analysis of genes and proteins, Edited by A D Baxevanis & B F F Ouellette, John Wiley & Sons, 1988. Any computer readable medium may be used, for example, compact disk, tape, floppy disk, hard drive or computer chips.

The polynucleotide sequences of the invention, or parts thereof, particularly those relating to and identifying the single nucleotide polymorphisms identified herein represent a valuable information source, for example, to characterise individuals in terms of haplotype and other sub-groupings, such as investigation of susceptibility to treatment with particular drugs. These approaches are most easily facilitated by storing the sequence information in a computer readable medium and then using the information in standard bioinformatics programs or to search sequence databases using state of the art searching tools such as "GCC". Thus, the polynucleotide sequences of the invention are particularly useful as components in databases useful for sequence identity and other search analyses. As used herein, storage of the sequence information in a computer readable medium and use in sequence databases in relation to 'polynucleotide or polynucleotide sequence of the invention' covers any detectable chemical or physical characteristic of a polynucleotide of the invention that may be reduced to, converted into or stored in a tangible medium, such as a computer disk, preferably in a computer readable form. For example, chromatographic scan data or peak data, photographic scan or peak data, mass spectrographic data, sequence gel (or other) data.

The invention provides a computer readable medium having stored thereon one or a more polynucleotide sequences of the invention. For example, a computer readable medium is provided comprising and having stored thereon a member selected from the group consisting of: a polynucleotide comprising the sequence of a polynucleotide of the invention,

a polynucleotide consisting of a polynucleotide of the invention, a polynucleotide which comprises part of a polynucleotide of the invention, which part includes at least one of the polymorphisms of the invention, a set of polynucleotide sequences wherein the set includes at least one polynucleotide sequence of the invention, a data set comprising or consisting of a polynucleotide sequence of the invention or a part thereof comprising at least one of the polymorphisms identified herein.

A computer based method is also provided for performing sequence identification, said method comprising the steps of providing a polynucleotide sequence comprising a polymorphism of the invention in a computer readable medium; and comparing said polymorphism containing polynucleotide sequence to at least one other polynucleotide or polypeptide sequence to identify identity (homology), i.e. screen for the presence of a polymorphism.

The invention will now be illustrated but not limited by reference to the following Examples. All temperatures are in degrees Celsius.

In the Examples below, unless otherwise stated, the following methodology and materials have been applied.

AMPLITAQ™, available from Perkin-Elmer Cetus, is used as the source of thermostable DNA polymerase.

General molecular biology procedures can be followed from any of the methods described in "Molecular Cloning - A Laboratory Manual" Second Edition, Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory, 1989).

Electropherograms were obtained in a standard manner: data was collected by ABI377 data collection software and the wave form generated by ABI Prism sequencing analysis (2.1.2).

Example 1

Identification of Polymorphisms

1. Methods

DNA Preparation

DNA was prepared from frozen blood samples collected in EDTA following protocol I (Molecular Cloning: A Laboratory Manual, p392, Sambrook, Fritsch and Maniatis, 2nd Edition, Cold Spring Harbor Press, 1989) with the following modifications. The thawed

blood was diluted in an equal volume of standard saline citrate instead of phosphate buffered saline to remove lysed red blood cells. Samples were extracted with phenol, then phenol/chloroform and then chloroform rather than with three phenol extractions. The DNA was dissolved in deionised water.

Template Preparation

Exons 5 and 7 were amplified from genomic DNA by PCR. Templates were prepared using the oligonucleotide primers described below.

Exon 5 was amplified in a two step PCR reaction with an annealing temperature of 68° and denaturation temperature of 94°. Exon 7 was amplified in a three step PCR reaction with an annealing temperature of 64°, extension temperature of 72° and denaturation temperature of 94°. Each step was 1 minute. Both reactions were carried out in 1.0mM $MgCl_2$ buffer.

For analysis generally 50 ng of genomic DNA was used in each reaction and subjected to 35 cycles of PCR.

Fragment	Forward Oligo 5'-3'	Reverse Oligo
Exon 5	ccagcctccatttctccagctg SEQ ID NO.1	ctggcaggtaacagtgacacca SEQ ID NO.2
Exon 7	caggcaacacctgtctacctg SEQ ID NO.3	gcaccgtcactgtctacttttttca SEQ ID NO.4

Forward oligos were modified by the addition of M13 forward sequence to the 5' end for use in dye-primer sequencing.

Dye Primer Sequencing

Dye-primer sequencing using M13 forward primer was as described in the ABI protocol P/N 402114 for the ABI Prism™ dye primer cycle sequencing core kit with "AmpliTaq FS"™ DNA polymerase, modified in that the annealing temperature was 45° and DMSO was added to the cycle sequencing mix to a final concentration of 5 %.

The extension reactions for each base were pooled, ethanol/sodium acetate precipitated, washed and resuspended in formamide loading buffer.

4.25 % Acrylamide gels were run on an automated sequencer (ABI 377, Applied Biosystems).

2. Results

Novel Polymorphisms

EMBL Sequence	Position	Published	Variant	RFLP	Frequency
L00394	41	C	T	eng Nco I	1/54
L00396	57	C	T	eng Spe I	39/48

Frequency is the allele frequency of the variant allele in control subjects.

"eng" = engineered RFLP

Example 2

Engineered restriction site primers for detection of polymorphisms

Standard methodology can be used to detect the polymorphism at position 41 (as defined by the position in EMBL ACCESSION NO L00394) and the polymorphism at position 57 (as defined by the position in EMBL ACCESSION NO. L00396) based on the materials set out below using a cDNA template.

EMBL Sequence	**Position**	**Diagnostic Fragment**	**Forward Oligo**	**Reverse Oligo**
L00394	41	17-156	17-40 Nco I	126-156
L00396	57	1-81	1-21	58-81 Spe I

Primer Sequence 5'-3'

17-40 Nco I ACGGAAGCTCTGCAGCCTGGACCA SEQ ID NO.5

58-81 Spe I TAGGATGTAGAACTCGCTCAGACT SEQ ID NO.6

T at position 41 generates an engineered Nco I site in the diagnostic fragment 17-156 described above. T at 57 generates an engineered Spe I site in the diagnostic fragment 1-81 as described above.

Sequence Listing Free Text

SEQ ID NO.1 <223>Description of Artificial Sequence: exon 5 forward primer

SEQ ID NO.2 <223>Description of Artificial Sequence: exon 5 reverse primer

SEQ ID NO.3 <223>Description of Artificial Sequence: exon 7 forward primer

SEQ ID NO.4 <223>Description of Artificial Sequence: exon 7 reverse primer

SEQ ID NO.5 <223>Description of Artificial Sequence: 17-40 Nco I primer

SEQ ID NO.6 <223>Description of Artificial Sequence: 58-81 Spe I primer